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(54) Title: LOW MOLECULAR WEIGHT OVERSULFATED POLYSACCHARIDE

(57) Abstract: LMW-K5-N,O-oversulfates are described, having a sulfation degree of from 3.2 to 4 and a mean molecular weight of from about 3,000 to about 6,000, obtainable by depolymerization of corresponding K5-N,O-oversulfates or starting from LMW-K5-N-sulfates by O-oversulfation of a tertiary amine or quaternary ammonium salt thereof and subsequent N-resulfation of the K5-amine-O-oversulfate thus obtained. Furthermore, pharmaceutical compositions containing these LMW-K5-N,Ooversulfates having antiangiogenetic and antiviral, in particular anti-HIV-1 activity. Intermediate LMW-K5-N-sulfates are also described.

LOW MOLECULAR WEIGHT OVERSULFATED POLYSACCHARIDE

SUMMARY OF THE INVENTION

The present invention refers to new N,O-oversulfated low molecular weight polysaccharides derived from K5 polysaccharide, to a process for their preparation, to new key intermediates in said process and to pharmaceutical compositions containing said oversulfated low molecular weight polysaccharides. More particularly, the present invention refers to a N-deacetylated and N-sulfated K5 having a degree of sulfation between 3.2 and 4 and a mean molecular weight from about 3,000 to about 6,000.

BACKGROUND OF THE INVENTION

Glycosamioglycans such as heparin, heparan sulfate, dermatan sulfate, chondroitin sulfate and hyaluronic acid are biopolymers industrially extracted from different animal organs.

In particular, heparin, mainly obtained by extraction from pig intestinal mucosa or from beef lung, is a polydisperse copolymer with a molecular weight distribution from about 3,000 to about 30,000 D formed by a mixture of chains essentially consisting of an uronic acid (glucuronic acid or iduronic acid) and of an aminosugar (glucosamine) linked by α-1$\rightarrow$ 4 or β-1$\rightarrow$ 4 bonds. In heparin the uronic unit can be O-sulfated in position 2 and the glucosamine unit is N-acetylated or N-sulfated, 6-O sulfated and 3-O sulfated in about 0.5 % of the glucosaminic units present.

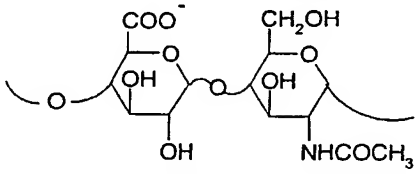
The properties and the natural biosynthesis of heparin in mammals were described by Lindahl et al., 1986 in Lane, D. and Lindahl, U. (Editors) "Heparin. Chemical and Biological Properties; Clinical Applications", Edward Arnold, London, Pages 159-190, by Lindhal, U, Feingold D. S. e Rodén L, 1986 TIBS, 11, 221-225 and by Conrad H. E. "Heparin Binding Proteins", Chapter 2: Structure of Heparinoids. Academic Press, 1998.

Besides the main anticoagulant and antithrombotic activities, heparin also exerts antilipemic, antiproliferative, antiviral, antitumoral and antimetastatic activity, but its use as a drug for such indications is hampered by the side effects due to its anticoagulant activity that can cause bleeding.

DESCRIPTION OF THE PRIOR ART

It is known that the capsular polysaccharide isolated from strains of Escherichia Coli (herein below also simply referred to as "K5") described by W.F. Vann et al. (1981) in Eur. J. Biochem 116, 359-364 consists of a mixture of chains formed by a repetitive disaccharide unit formed by D-glucuronic acid and N-

acetylglucosamine linked β1→4, while the disaccharide units D-glucuronyl-N-acetylglucosamine are linked α 1→4, thus showing the same repeated sequence (A)



(A)

as the N-acetylheparosan biosynthetic precursor of heparin and heparan sulfate. The only difference, which is irrelevant for the biological activities of the K5 and its derivatives, between the heparin precursor N-acetylheparosan and K5 polysaccharide, is the presence of a double bond in position 4(5) at the non reducing end of some chains of the polymer, as for instance described in EP 489647 and EP 544592 mentioned herein below.

After these first publications, other papers and patent applications described the preparation of the E.coli K5 polysaccharide having molecular weight ranges from few thousands to many hundred thousand Daltons. For example EP 333243, IT 1230785, EP 489647, EP 544592, WO 92/17507, WO 01/02597, and the paper by M. Manzoni et al. (1996), Biotechnology Letters, 18(4) 383-386 are cited.

Documents EP 489647 and EP 544592 disclose low molecular weight and high molecular weight N,O sulfate heparosans having anticoagulant and antithrombotic activity, IT 1230785, WO 92/17507, WO 96/14425, WO 97/43317, WO 98/42754, WO 01/72848 and US 2002/0062019 describe derivatives of N-deacetylated-K5-N-sulfate, having a certain number of glucuronic units epimerized in position C5 to iduronic units, with antithrombotic activity and WO 98/09636 describes N-deacetylated-K5-N-sulfate with antimetastatic activity.

The document US 2002/0062019 describes a process for the preparation of epiN,O-sulfated-K5 derivatives, active on the coagulation control, having a degree of sulfation of from 2.3 to 2.9 and a molecular weight of from 2,000 to 30,000, or from 4,000 to 8,000 or from 18,000 to 30,000. Said process comprises the steps (s-a) a N–deacetylation of K5 polysaccharide and a N-sulfation of the resulting K5-amine, (s-b) an epimerization of the N-sulfate-K5, (s-c) an O-oversulfation of the epiK5-N-sulfate, (s-d) a partial O-desulfation, (s-e) a selective 6-O-sulfation, (s-f) a N-sulfation of the so obtained product, whatever product obtained at the end of steps (s-b)-(s-f) being optionally submitted to a depolymerization. Said document describes a N-sulfated-epiK5 having a molecular weight of 7,400 and a degree of sulfation of

from 2.3 to 2.9, obtained by the above-mentioned steps (s-a)-(s-f) followed by a nitrous depolymerization at the end of the (s-f) step.

The same document describes a K5 fraction with a molecular weight of about 5,000 that can also be submitted to the (s-a)-(s-f) steps.

The pending Italian patent application n. MI2001A/00397 (WO 02/068477), incorporated into the present application as a reference, describes K5-N,O-oversulfate-derivatives having a degree of sulfation higher than 3.2, obtained starting from a K5 free from lipophilic substances or from one of its fractions with a molecular weight of about 5,000 by (a) N-deacetylation/N-sulfation, (b) O-oversulfation, and (c) N-resulfation. This document cites LMW-K5-N,O-oversulfate having a mean molecular weight of from 2,000 to 5,000 obtained by depolymerization of the K5-N,O-oversulfate or a LMW-K5-N,O-oversulfate of mean molecular weight of about 6,500 directly obtained from the above mentioned fraction of K5 by the steps (a)-(c).

None of the above cited documents describe LMW-K5-N-sulfate, optionally 40%-60% epimerized, in which NH2 or N-acetyl groups are practically absent.

Moreover, D.Leali et al., in a paper titled "Fibroblast Growth Factor 2 Antagonist Activity and Angiostatic Capacity of Sulfated Escherichia coli K5 Polysaccharide Derivatives" published in J. Biol. Chem 2001 (October 12), 276(41), 37900-37908 (Leali 2001), described a K5-N,O-oversulfate having a mean molecular weight of 15,000 and a degree of sulfation of 3.84, which shows a good antiangiogenic activity expressed as 70% of inhibition of the formation of new vessels within the 12th day of incubation.

To make the terminology uniform and the text more understandable, in the present description meanings and conventional expressions, in singular or plural form, will be used. In particular:

- "K5" or "polysaccharide K5" means the capsular polysaccharide from Escherichia coli obtained by fermentation, namely a mixture of chains consisting of repetitive disaccharide units A, optionally containing a double bond at the non reducing end as illustrated above, whenever prepared and purified according to the methods described in the literature, in particular according to Vann 1981, Manzoni M. et al., Journal of Bioactive and Compatible Polymers, 1996, 11, 301-311 ("Manzoni 1996"), according to the method described in WO 01/72848 or in example 12 of US 2002/0062019 A1; it is obvious for a skilled in the art that the matter illustrated herein is applicable to any N-acetylheparosan;

- "K5 amine" means the K5, N-deacetylated for at least 95%, but generally in which acetyl groups are undetectable by a current NMR apparatus;
- "K5-N-sulfate" means the K5, N-deacetylated and N-sulfated for at least 95% as herein below described, but generally in which acetyl groups are undetectable by a current NMR apparatus;
- "K5-amine-O-oversulfate" means an O-sulfated-K5-amine with a degree of sulfation of at least 2.2;
- "K5-N,O-oversulfate" means a N,O-sulfated-K5-amine with a degree of sulfation of at least 3.2.

Moreover:

- the terms and conventional expressions herein above defined refer to K5 polysaccharides as isolated after fermentation, generally with a distribution of molecular weight from about 1,500 to about 50,000 with a mean molecular weight of 12,000-25,000, advantageously of 15,000-25,000;
- apart from the specific attribution of the molecular weight, the terms and conventional expressions herein above defined, when preceded by the acronym "LMW" (low molecular weight), indicate low molecular weight products having a mean molecular weight up to12,000;
- "about" referred to the molecular weight means the molecular weight measured by viscosimetry ± the theoretical weight of a disaccharide unit, including the weight of sodium, calculated to be 461 in the case of a K5-N-sulfate and 765 in the case of a K5-N,O-oversulfate with a degree of sulfation of 3.87;
- the terms and conventional expressions as herein above defined, when are followed by "-derivative" globally designate both the derivatives from native K5 and those of low molecular weight, independently of the fact that these are obtained by fractionation of K5 or of its derivatives or by depolymerization;
- unless otherwise specifically indicated, "degree of sulfation" means the SO3-/COO- ratio, that can be expressed also as the number of sulfate groups per disaccharide unit, as measured by the conductimetric method described by Casu B. et al. in Carbohydrate Research, 1975, 39, 168-176 (Casu 1975);
- unless otherwise specifically indicated, the molecular weight is intended to be measured by viscosimetry according to Johnson et al. Carb. Res. n.51 (1976) p. 119-127, using samples whose molecular weight was calculated by HPLC as standards;

- "preponderant species" means the compound that, in the mixture constituting the LMW-K5-N-sulfate, the K5-amine-O-oversulfate or the LMW-K5-N,O-oversulfate, is the most represented species, determined by the peak of the molecular weight curve measured by HPLC;
- "O-oversulfation conditions" means an O-sulfation performed for example according to Method C described by B. Casu et al. in Carbohydrate Research, 1994, 263, 271-284 (Casu 1994);
- the term "alkyl" means a linear or branched alkyl, while "tetrabutylammonium" means the tetra(n-butyl)ammonium group.

SUMMARY OF THE INVENTION

It has now been found that it is possible to depolymerize a K5-N-sulfate to obtain new LMW-K5-N-sulfate derivatives which constitute useful starting materials for the preparation of new LMW-K5-N,O-oversulfates. Advantageously, it is also possible to obtain new LMW-K5-N-sulfates with very low mean molecular weight, in particular from about 2,000 to about 4,000, more particularly specific LMW-K5-N-sulfates formed by mixtures in which the preponderant compound is a decasaccharide or a dodecasaccharide or a tetradecasaccharide. These new LMW-N-sulfates are useful intermediates for the preparation of LMW-K5-N,O-oversulfates with antiviral and/or antiangiogenic activity and devoid of anticoagulant activity.

More particularly, it has been found that a LMW-K5-N,O-oversulfate having a mean molecular weight of from about 3,000 to about 6,000, in particular a LMW-K5-N,O-oversulfate consisting of a mixture in which the preponderant species is a deca-, dodeca- or tetradecasaccharide, with a degree of sulfation of from 3.2 to 4, has an antiangiogenic activity higher than that of the K5-N,O-oversulfate described by Leali 2001.

It has also been found that said new LMW-K5-N,O-oversulfates having a mean molecular weight from about 3,000 to about 6,000, in particular LMW-K5-N,O-oversulfates consisting of a mixture in which the preponderant species is a deca-, dodeca- or tetradecasaccharide, avoid of anticoagulant activity, have a good activity on HIV-1 virus.

Moreover it has been found that, starting from said LMW-K5-N-sulfates, it is possible to obtain new LMW-O-sulfated-K5-amine with a high degree of sulfation by preparing a tertiary amine or quaternary ammonium salt of said LMW-K5-N-sulfate, taking care to keep the reaction mixture for a period of time of 30-60 minutes by maintaining the pH at about 7, and then by treating the obtained salt with an O-

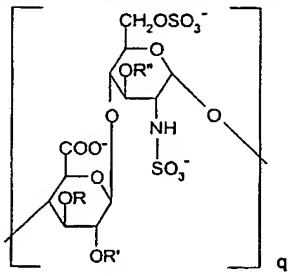
sulfating reactant in the conditions of O-oversulfation. By N-sulfating the above mentioned LMW-K5-amine-O-oversulfate, new LMW-K5-N,O-oversulfate are obtained.

DETAILED DESCRIPTION OF THE INVENTION

Thus, it is an object of the present invention to provide new LMW-K5-N,O-oversulfate having a mean molecular weight of from about 3,000 to about 6,000 and a degree of sulfation of from 3.2 to 4, advantageously from 3.5 to 4, preferably from 3.7 to 3.9.

Among these new LMW-K5-N,O-oversulfate of the present invention, particularly interesting are those having a mean molecular weight of 3,750-4,250, of 4,750-5,250 or of 5,750-6,250.

Preferential compounds are LMW-K5-N,O-oversulfates formed by a mixture of chains in which the preponderant species has the formula I



(I)

in which q is 4, 5, 6, 7 or 8 and R, R' and R'' represent hydrogen or a SO3- group, for a degree of sulfation of from 3.2 to 4, advantageously from 3.5 to 4, preferably from 3.5 to 3.9 and the corresponding cation is a chemically or pharmaceutically acceptable one.

In this context, the term "chemically acceptable" refers to a cation useful in the chemical syntheses, such as sodium, ammonium, tetra(C_1-C_4)alkylammonium, or for the purification of the product, while "pharmaceutically acceptable" is self explanatory.

Advantageous cations are those derived from alkaline metals, earth-alkaline metals, ammonium, tetra(C_1-C_4)alkylammonium, aluminum and zinc.

The LMW-K5-N,O-oversulfate of the present invention can be prepared by depolymerization of K5-N,O-oversulfates of the type described in Leali 2001 and prepared with the method described in it.

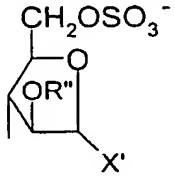
Said depolymerization is performed according to the known methods for the depolymerization of heparin, for instance according to the method described in EP 37319, in WO 82/03627 or the method for the depolymerization of a N,O-sulfated-

K5 described in EP 544592. Preferably, the depolymerization, performed with sodium nitrite and hydrochloric acid ("nitrous depolymerization"), is followed by an in situ reduction with sodium borohydride.

By suitably controlling the depolymerization reaction, in particular by using different amounts of sodium nitrite, LMW-K5-N,O-oversulfates are obtained having the desired molecular weight.

According to a preferred embodiment, starting for instance from 1 g of K5-N,O-oversulfate obtained as described in PREPARATION IV herein below, the starting product is dissolved in 100-200 ml of deionized water and thermostated at 4°C. Then an amount of sodium nitrite to obtain the desired mean molecular weight from about 3,000 to about 6,000 is added. Consequently, starting from a K5-N,O-oversulfate having a molecular weight of 20,000 measured by HPLC equipped with a BioSil 250 Biorad column and using standards of heparin with known molecular weight, from 160 to 230 mg of sodium nitrite dissolved in a water solution at 0.2% shall be added. The solution containing the K5-N,O-oversulfate and sodium nitrite, kept at 4°C, is brought to pH 2 by adding 0.1N HCl cooled at 4°C. The reaction is left to react for 20-40 minutes, then neutralized with 0.1 N NaOH. The obtained product is brought to room temperature and treated with a reducing agent such as sodium borohydride (250-500 mg dissolved in 50-100 ml of water) and left to react for 4-8 hours. The excess of sodium borohydride is eliminated by bringing the pH to 5-5.5 with 0.1N HCl and left to react for further 2-4 hours. At the end, the solution is neutralized with 0.1N NaOH and the product is recovered by precipitation with acetone or ethanol after concentration of the product by evaporation under reduced pressure.

The origin of the LMW-K5-N,O-oversulfates from the nitrous depolymerization of a K5-N,O-oversulfate and subsequent possible reduction with for instance sodium borohydride implies, at the reducing end of the majority of the chains in said mixture of chains, the presence of a sulfated 2,5-anhydromanno unit having the structure (a°)

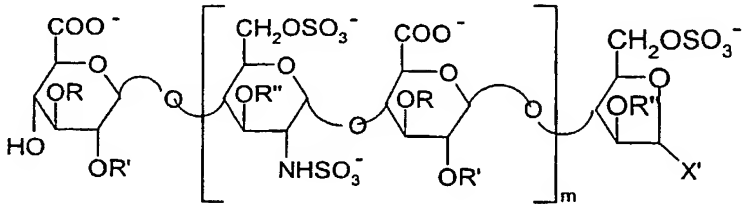


(a°)

in which X' represents formyl or hydroxymethyl and R" represents hydrogen or SO_3^-.

In particular, preferred products are the LMW-K5-N,O-oversulfate, obtainable by nitrous depolymerization of a K5-N,O-oversulfate and optional subsequent reduction

with, for instance, sodium borohydride, consisting of mixtures of chains in which the preponderant species is a compound of formula I°



(I°)

wherein m is 4, 5 or 6, R,R' and R" are hydrogen or SO_3^-, X' is formyl or hydroxymethyl, for a degree of sulfation of from 3.2 to 4, advantageously from 3.5 to 4, preferably from 3.5 to 3.9 and the corresponding cation is a chemically or pharmaceutically acceptable one. The mean molecular weight of each mixture advantageously is 3,750-4,250, 4,750-5,250, 5,750-6,250.

These new LMW-K5-N,O-oversulfates have high antiangiogenic activity with a favourable ratio to the global anticoagulant activity and can be used in the preparation of pharmaceutical compositions for the treatment of angiogenesis-dependent pathologies in doses in which the risk of hemorragic side effects is extremely reduced.

Angiogenesis dependent pathologies that can be treated with the LMW-K5-N,O-oversulfates are for example, among those found in the human beings, diabetic retinopathy, neovascularization of the transplanted cornea, neovascular glaucoma, trachoma, retrolental fibrodisplasia, psoriasis, pyogenic glaucoma, development of the ateroschlerotic plaque, hemangioma and angiofibroma, artero-venous malformations, arthritis, and in the combinatorial therapy of solid tumors.

More particularly, the new LMW-K5-N,O-oversulfates of the present invention were active in the in vivo test of the inhibition of the angiogenesis on chicken embryo choroallantioic membrane (CAM) according to Ribatti D. et al., J. Vasc. Res. 1997, 34, 455-463 (Ribatti 1997). According to this test, sponge implants of Gelfoam (Upjohn) are applied on the CAM of chicken embryos at the 8th day of development and, immediately after the application, 3 µl of a solution of physiologic saline containing 50 µg of LMW-K5-N,O-oversulfate are applied or, as reference compound, of K5-N,O-oversulfate with 15,000 molecular weight and degree of sulfation of 3.84 described in Leali 2001. The sponges are examined every day till the 12th day of incubation. The score of angiogenesis is obtained by counting the number of macroscopic vessels observable around the sponge at the different days of development and at the number of embryos (eggs) on which the compound is active.

It was observed that the number of vessels that grow around the sponge embedded of LMW-K5-N,O-oversulfate is equal to those formed around the sponge embedded of K5-N,O-oversulfate, but the number of embryos on which the LMW-K5-N,O-oversulfate is active is higher.

LMW-K5-N,O-oversulfates having a mean molecular weight of from about 3,000 to about 6,000 can be also prepared according to a process that is not described in literature and that is generally applicable to the preparation of new LMW-K5-N,O-oversulfates having a mean molecular weight from about 3,000 to about 12,000. Said new process and said new LMW-K5-N,O-oversulfates represent further aspects of the present invention.

Thus the present invention also provides a process for the preparation of new LMW-K5-N,O-oversulfates having a degree of sulfation of from 3.2 to 4, advantageously from 3.5 to 4, preferably from 3.5 to 3.9, which comprises

(a) treating a LMW-K5-N-sulfate, in its acidic form, with a tertiary amine or quaternary ammonium hydroxide, letting the reaction mixture to stand for a period of time of 30-60 minutes, whereby the pH of the solution is maintained at 7, and isolating the salt with said organic base;

(b) treating the tertiary amine or quaternary ammonium salt of said LMW-K5-N-sulfate thus obtained with an O-sulfation reactant under O-oversulfation conditions;

(c) treating the product thus obtained with a N-sulfating agent and isolating the obtained LMW-K5-N,O-oversulfate. Usually the final product is isolated as sodium salt that is optionally transformed in another chemically or pharmaceutically acceptable salt.

A LMW-K5-N-sulfate obtained from a K5 by a practically complete N-deacetylation, subsequent N-sulfation, and final nitrous depolymerization followed by reduction of the LMW-K5-N-sulfate, as above illustrated, is used as starting material for the process of the present invention. Said reduction is necessary since the LMW-K5-N-sulfate subsequently undertakes reactions wherein the influence on the formyl group of the 2,5 anhydromannose radical is unknown. Also in this case, by controlling the depolymerization reaction as above illustrated, LMW-K5-N-sulfate can be obtained, having a mean molecular weight in the whole range from about 1,500 to about 10,000, preferably from about 1,500 to about 7,500, as calculated from the 13C-NMR spectrum by the integration of the signal attributable

to the C-2 of 2,5 anhydromannitol with that of the anomeric carbon of the glucosamine inside the polysaccharide chain.

LMW-K5-N-sulfates having a mean molecular weight from about 1,500 to about 7,500 and their chemically or pharmaceutically acceptable salts are new products useful as intermediates and also pharmaceutically active. The molecular weight distribution of said LMW-K5-N-sulfates can be from about 1,000 to about 10,000.

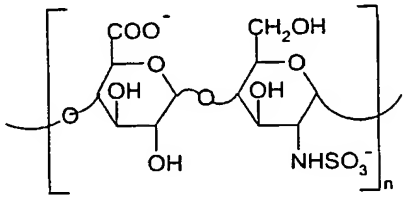
According to a general way to proceed, starting for instance from 1 g of K5-N-sulfate, the starting product is dissolved in 100-200 ml of deionized water and thermostated at 4°C. Then an amount of sodium nitrite to obtain the desired mean molecular weight of from about 2,000 to about 4,000 is added. Consequently, starting from a K5-N-sulfate having a molecular weight of 20,000 measured by HPLC equipped with a BioSil 250 Biorad column and using standards of heparin with known molecular weight, from 330 to 480 mg of sodium nitrite dissolved in a 0.2% water solution shall be added. The solution containing the K5-N-sulfate and sodium nitrite, kept at 4°C is brought to pH 2 by adding 0.1N HCl cooled at 4°C. The reaction is left to react for 20-40 minutes, then neutralized with 0.1 N NaOH. The obtained product is warmed up to room temperature and treated with a reducing agent such as sodium borohydride (250-500 mg dissolved in 50-100 ml of water) and left to react for 4-8 hours. The excess of sodium borohydride is eliminated by bringing the pH to 5-5.5 with 0.1N HCl and left to react for further 2-4 hours. At the end the solution is neutralized with 0.1N NaOH and the product is recovered by precipitation with acetone or ethanol after concentration of the product by reduced pressure evaporation.

Analogously, the amount of sodium nitrite that, starting from 1 g of K5-N-sulfate, allows to obtain a LMW-K5-N-sulfate with a molecular weight of from about 4,000 to about 7,500, in particular of at least 6,000 (6,000-7,500), can be established.

The K5-N-sulfate is very well known in the literature and it is described in the documents herein above cited to illustrate the state of the art. The above cited starting material is invariably obtained by N-deacetylation and subsequent N-sulfation of the thus obtained K5-amine. However, it was noted that the preparation of a K5-N-sulfate practically devoid of acetyl groups or NH2 is facilitated if the K5 from which it is prepared is particularly pure, in particular if it does not contain lipophilic substances. Moreover, it was found that a K5-N-sulfate prepared from a K5 free from lipophilic substances is easier oversulfated, as described in the pending patent application IT MI2001A00397 (WO 02/068477). It is then preferable that the

depolymerization is performed starting from a K5-N-sulfate prepared from a K5 purified as described in PREPARATION I herein below. Said K5-N-sulfate, whose ^{13}C-NMR spectrum does not show traces of acetyl groups or NH2, is described in PREPARATION II herein below.

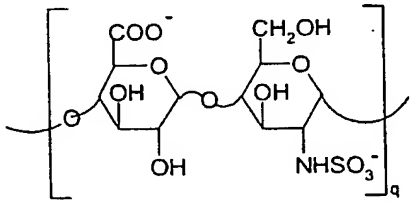
Advantageous starting materials of the process of the present invention are new LMW-K5-N-sulfates obtained by nitrous depolymerization of a K5 and subsequent reduction, for instance with sodium borohydride, consisting of mixtures of chains in which at least 90% of said chains have the formula II



(II)

wherein n represents a number from 2 to 20 and the corresponding cation is a chemically or pharmaceutically acceptable one.

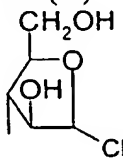
More advantageously, the starting materials are new LMW-K5-N-sulfates consisting of a mixture of chains in which the preponderant species has the formula II'



(II')

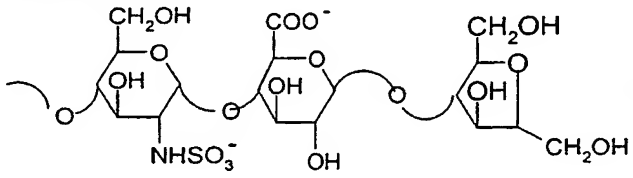
wherein q is 4, 5, 6, 7, or 8 and the corresponding cation is a chemically or pharmaceutically acceptable one. These LMW-K5-N-sulfates, that constitute an advantageous aspect of the invention, are obtained from a K5-N-sulfate by nitrous depolymerization and subsequent reduction for instance with sodium borohydride as illustrated above. Their mean molecular weight is from about 2,000 to about 4,000.

The origin of the LMW-K5-N-sulfates from a nitrous depolymerization step and subsequent reduction with for instance sodium borohydride, implies the presence of a 2,5 anhydrommanitol unit of structure (a')



(a')

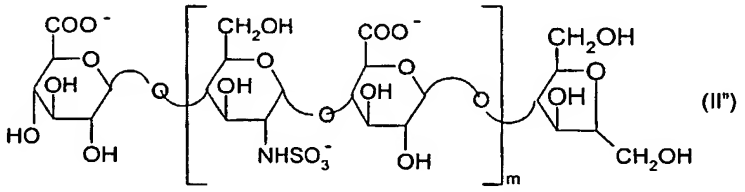
at the reducing end of the majority of the chains in said mixture of chains. Consequently, the reducing terminal of the majority of the chains is actually represented by the structure (b)



(b)

The presence of the structure (a') has no influence on the properties of the LMW-K5-N-sulfates and of their derivatives because possible sulfations would only involve a possible introduction of one or more sulfate groups that would not significantly influence the degree of sulfation of the O-sulfated derivatives. The preferred LMW-K5-N-sulfates are practically devoid of acetyl groups.

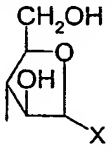
Particularly advantageous LMW-K5-N-sulfates according to the present invention are formed by mixtures of chains in which the preponderant species is a compound of formula II'



(II")

in which m represents 4, 5 or 6 and the corresponding cation is a chemically or pharmaceutically acceptable one.

According to another of its aspects, the invention relates to a process for the preparation of new LMW-K5-N-sulfates and of their chemically or pharmaceutically acceptable salts, which comprises submitting a K5-N-sulfate to a controlled nitrous depolymerization optionally followed by a reduction and isolating the product so obtained. Said products usually are in form of their sodium salt, that can be transformed into another chemically or pharmaceutically acceptable salt. At the reducing end of the majority of the chains by which they are composed, said LMW-K5-N-sulfates have a 2,5-anhydromanno unit having the structure (a)



(a)

in which X represents a formyl or hydroxymethyl group and, preferably, they are formed by mixtures of chains in which at least 90% of said chains has the formula II or by mixtures of chains in which the preponderant species has the formula II' or II".

These new LMW-K5-N-sulfates can be used as active ingredients of pharmaceutical compositions.

If, in the structure (a) above, X represents hydroxymethyl, the new LMW-K5-N-sulfates are the starting material of the process for the preparation of the LMW-K5-N,O-oversulfates of the present invention.

Advantageously, the LMW-K5-N-sulfate has a molecular weight distribution from about 1,000 to about 10,000.

The LMW-K5-N-sulfates starting materials of the process of the present invention are preferably used as sodium salt, unless a tertiary amine or quaternary ammonium salt prepared according to step (a) above illustrated, preferably the tetrabutylammonium salt, is already available.

According to another advantageous embodiment of the process of the present invention, step (a) is performed by passing a solution of the sodium salt of the starting LMW-K5-N-sulfate through an acid ionic exchange resin, for example of the type IR-120 H+, by collecting the eluate comprising also the waste of the resin and by neutralizing the eluate with a tertiary amine or quaternary ammonium hydroxide, preferably with an aqueous solution of tetrabutylammonium hydroxide. The solution is let to stand for one hour, by concurrently maintaining the pH at 7 by adding the same base and the so obtained salt is isolated by freeze drying.

In step (b), the O-oversulfation is performed using an excess of O-sulfating agent at a temperature from 20 to 70°C for a period of time up to 24 hours in a polar aprotic solvent. Advantageously, the tertiary amine or quaternary ammonium salt of the LMW-K5-N-sulfate so obtained in step (a), is dissolved in dimethylformamide and treated with 2-10 moles of an O-sulfating agent for each free hydroxy group at a temperature of 40-60°C for 10-20 hours. As O-sulfating agent, the adduct pyridine.SO_3 is advantageously used, in amounts of 2.5-5 moles, preferably 2.5-4 moles per free hydroxy group and the reaction is advantageously performed at 50-60°C, preferably at 55°C overnight. The product obtained at the end of the reaction is isolated by addition of 0.1-1 volume of water and neutralization, preferably with sodium hydroxide, by precipitation with a saturated solution of sodium chloride in acetone and filtration optionally followed by an ultrafiltration.

The so obtained product is preferably the sodium salt of a LMW-K5-amine-O-oversulfate having a degree of sulfation of from 2.3 to 3. The sodium salt so obtained can be converted into another salt. The mean molecular weight of such a product can be from about 3,500 to about 11,000.

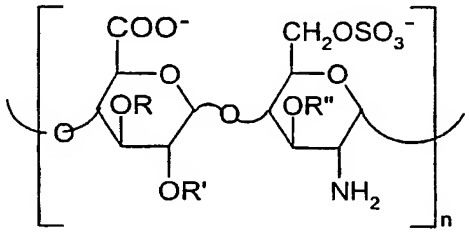
In step (c), the LMW-K5-amine-O-oversulfates are submitted to a N-sulfation, performed by treating their aqueous solution with sodium carbonate and a N-sulfating agent, for example a (C_1-C_4)trialkylamine.SO_3 or pyridine.SO_3, by maintaining the mixture at 30-50°C for 8-24 hours and by isolating the LMW-K5-N,O-oversulfate for example by diafiltration.

The LMW-K5-amine-O-oversulfates and their chemically or pharmaceutically acceptable salts obtainable according to the above said process, limited to steps (a) and (b), are new products which constitute a further aspect of the present invention. Surprisingly, it was found that LMW-K5-amine-O-oversulfates, besides being useful intermediates, also have pharmacobiological activities.

Thus, the present invention also provides new LMW-K5-amine-O-oversulfates having a degree of sulfation of from 2.3 to 3 and their chemically or pharmaceutically acceptable salts. Advantageously, their mean molecular weight is from about 3,500 to about 10,000, more advantageously from about 3,500 to about 5,200.

Preferably they are substantially free of N-acetyl groups.

If a LMW-K5-N-sulfate consisting of a mixture of chains in which at least 90% of said chains has the above mentioned formula II is used as starting material, a new LMW-K5-amine-O-oversulfate consisting of a mixture of chains in which at least 90% of said chains have the formula III

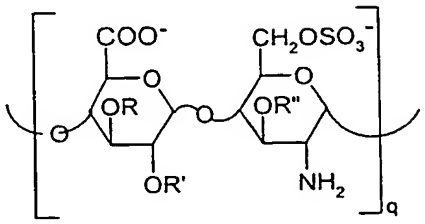


(III)

in which n is an integer from 2 to 20, R, R' and R" represent a hydrogen or a SO3-group, for a degree of sulfation of from 2.2 to 3, and the corresponding cation is a chemically or pharmaceutically acceptable one, is obtained at the end of step (b).

If an advantageous LMW-K5-N-sulfate consisting of a mixture of chains in which the preponderant species has the formula II' is used as starting material, a new

LMW-K5-amine-O-oversulfate consisting of a mixture of chains in which the preponderant species has the formula III'

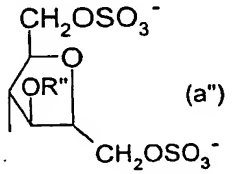


(III')

in which q is 4, 5, 6, 7, or 8, R, R' and R" are as defined above, the degree of sulfation is from 2.3 to 3, and the corresponding cation is a chemically or pharmaceutically acceptable one, is obtained at the end of step (b).

These LMW-K5-amine-O-oversulfates are new products useful as intermediates in the preparation of their N-sulfated derivatives, but they have per se interesting pharmacological properties, in particular against free radicals.

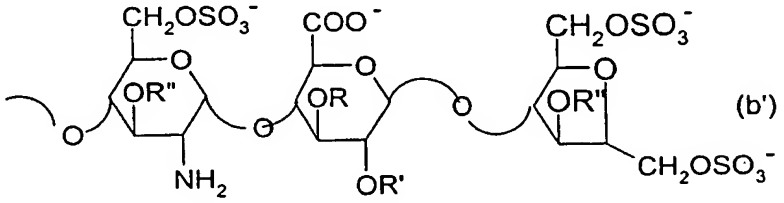
The origin of the new LMW-K5-amine-O-oversulfates from LMW-K5-N-sulfates obtained by nitrous depolymerization and subsequent reduction with, for instance, sodium borohydride, involves the presence of a 2,5 anhydromannitol sulfated unit of structure (a")



(a")

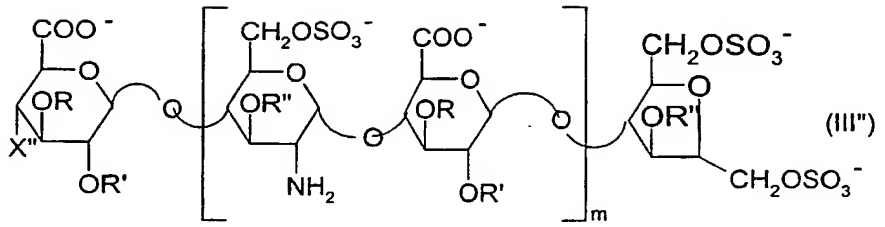
in which R" represents hydrogen or SO_3^-, at the reducing end of the majority of the chains in said mixture of chains.

Thus, the reducing end of the majority of the chains in said mixture of chains is represented by the structure (b')



(b')

Among the new above mentioned LMW-K5-amine-O-oversulfates, those consisting of mixtures in which the preponderant species is a compound of formula (III")



(III")

wherein R, R', R" are hydrogen or SO_3^-, X" is OH or OSO_3^-, for a degree of sulfation of from 2.2 to 3, m is 4, 5, or 6 and the corresponding cation is a chemically or pharmaceutically acceptable one, are preferred.

According to the present invention, starting from a K5-N-sulfate it is possible to prepare LMW-K5-N,O-sulfates and their chemically or pharmaceutically acceptable salts by a process which comprises

(i) submitting a K5-N-sulfate to a nitrous depolymerisation followed by a reduction, for example by sodium borohydride;

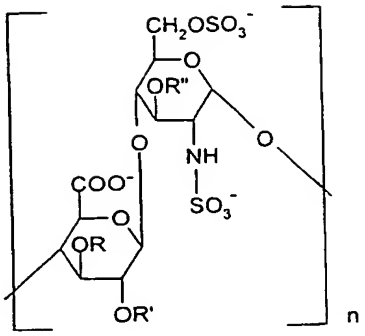
(ii) treating a LMW-K5-N-sulfate, in its acidic form, with a tertiary amine or quaternary ammonium hydroxide, letting the reaction mixture to stand for a period of time of 30-60 minutes, whereby the pH of the solution is maintained at 7, and isolating the corresponding tertiary amine or quaternary ammonium salt;

(iii) treating said tertiary amine or quaternary ammonium salt of said LMW-K5-N-sulfate with an O-sulfation reactant under O-oversulfation conditions;

(iv) treating the product thus obtained with a N-sulfating agent and isolating the obtained LMW-K5-N,O-oversulfate.

The new LMW-K5-N,O-oversulfates obtained at the end of the process of the present invention are generally present as sodium salt. Said sodium salt can be converted into another chemically or pharmaceutically acceptable salt. For example, an exchange with the calcium ion can be performed using ultrafiltration membranes. Particularly advantageous salts are those of alkaline metals, earth-alkaline metals, ammonium, tetra(C_1-C_4)alkylammonium, aluminum, and zinc. The sodium, calcium and tetrabutylammonium are preferred salts.

The LMW-K5-N,O-oversulfates obtained according to the process of the present invention consist of mixtures of chains in which at least 90% of said chains has the structure I'

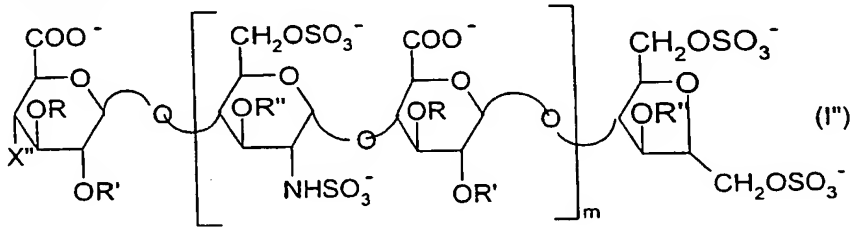


(I')

in which n is an integer from 2 to 20, R, R' and R" represent hydrogen or a SO_3^- group, in which the reducing end of the majority of said chains has the structure (a"), for a degree of sulfation of from 3.2 to 4, advantageously from 3.5 to 4, preferably from 3.5 to 3.9, and the corresponding cation is a chemically or pharmaceutically acceptable one.

Among these new LMW-K5-N,O-oversulfates obtained according to the process of the present invention those consisting of mixtures of chains in which the preponderant species has the formula I and in which the reducing end of the majority of said chains has the structure (a") are particularly advantageous.

Preferred LMW-K5-N,O-oversulfates among those obtained according to the process of the present invention consist of mixtures of chains in which the preponderant species is a compound of formula I"



(I")

wherein R, R' and R" are hydrogen or SO3-, X" is OH or OSO3-, for a degree of sulfation of from 3.2 to 4, advantageously from 3.5 to 4, preferably from 3.5 to 3.9, m is 4, 5 or 6 and the corresponding cation is a chemically or pharmaceutically acceptable one.

The new LMW-K5-N,O-oversulfates of the present invention are interesting as active principles useful in therapy since they possess antiviral, especially anti HIV-1, activity and an excellent antiangiogenic activity. Particularly interesting are the LMW-K5-N,O-oversulfates with a molecular weight of 3,750-4,250 (m = 4), of 4,750-5,250 (m = 5) or of 5,750-6,250 (m = 6) and a degree of sulfation of from 3.5 to 3.9.

All the LMW-K5-N,O-oversulfates above illustrated, due to their antiviral and antiangiogenic activity, constitute interesting drugs for the treatment of the above mentioned pathologies. For the intended therapeutic uses, the active principles of the present invention and their salts will be formulated according to conventional techniques in suitable forms of administration such as for example sterile solutions, topic dosage forms and, in general, in all of those forms proposed until today for glycosaminoglycan-type derivatives. Also the therapeutic dosages will be chosen in analogy with those already studied for the known natural compounds.

The administration of the active principle can be performed by oral, transdermic or preferably parenteral, in particular subcutaneous, intramuscular or intravenous, or topic route.

In man, the intended daily dosage for the parenteral administration is of 0.5-500mg/Kg/die, advantageously of 5-250 mg/kg/die, preferably of 10-150 mg/kg/die, while the dosage intended for the topical route is of 1-1,000 mg/Kg/die, advantageously 10-500 mg/Kg/die, preferably 20-100 mg/Kg/die.

Thus, according to another of its aspects, the present invention provides a pharmaceutical composition comprising, as an active ingredient, a pharmacologically effective amount of a LMW-K5-N,O-oversulfate advantageously consisting of mixture of chains in which the preponderant species is a compound of formula I, I°, I" or by mixtures of chains in which at least 90% of said chains has the formula I', for a degree of sulfation of from 3.2 to 4, advantageoulsy from 3.5 to 4, preferably from 3.5 to 3.9, or of its pharmaceutically acceptable salts, in admixture with a pharmaceutical vehicle. Said LMW-K5-N,O-oversulfates and their salts are largely illustrated herein above. Advantageous pharmaceutically acceptable salts are sodium, potassium, calcium, magnesium, aluminum and zinc salts.

In the pharmaceutical compositions of the present invention for oral, subcutaneous, intravenous, transdermic or topic administration, the active ingredients are preferably administered as dosage units, in admixture with the classic excipients or pharmaceutical vehicles. The dose can amply change in function of age, weight, and health conditions of the patient, as much as of severity of the infection and of route of administration. This dose comprises the administration of a dosage unit of from 1 to 1,000 mg, advantageously from 10 to 750 mg, preferably from 250 to 500 mg, once to three times per day, by intravenous, intramuscular, subcutaneous, oral, transdermic, transmucosal or topical route.

The pharmaceutical compositions of the present invention are formulated with the pharmaceutical carriers suitable for the various administration routes. Formulations in form of cream, pomade, ointment, gel, intravaginal ovules, suppositories, solution or suspension adapted to local administration.

In particular, the present invention provides a pharmaceutical composition for the treatment of angiogenesis dependent pathologies or for the treatment of HIV infection which comprises, as an active ingredient, a pharmacologically effective amount of a LMW-K5-N,O-oversulfate advantageously consisting of mixtures of chains in which the preponderant species is a compound of formula I, I°, I'' or by mixtures of chains in which at least 90% of said chains has the formula I', for a degree of sulfation of from 3.2 to 4, advantageously from 3.5 to 4, preferably from 3.5 to 3.9, or of one of its pharmaceutically acceptable salts, in admixture with a pharmaceutical vehicle or carrier.

Finally, the present invention relates to a pharmaceutical composition comprising, as one of its active ingredients , a new LMW-K5-amine-O-oversulfate obtainable according to steps (a) and (b) of the process above described, especially a LMW-K5-amine-O-oversulfate having a degree of sulfation of from 2.2 to 3, advantageously having a mean molecular weight from about 3,500 to about 11,000, more advantageously from about 3,500 to about 5,200, preferably consisting of a mixture of chains in which at least 90% of said chains has the formula III or in which the preponderant species is a compound of formula III' or III'', or one of its pharmaceutically acceptable salts, in admixture with a pharmaceutical carrier. Preferably said LMW-O-oversulfated-K5 amine with a degree of sulfation of from 2.2 to 3 is substantially free of N-acetyl groups.

The following examples illustrate the invention without, however, limiting it.

PREPARATION I

One gram of K5 obtained as described in paragraphs [0251] and [0252] of example 12 of US 2002/0062019, giving a ^{1}H-NMR spectrum (figure 3) in which signals due to lipophilic substances are present in the region below 1.5 ppm, is dissolved in 100 ml of a saturated aqueous solution of sodium chloride, thermostated at 4°C. To the so obtained solution, 3 volumes of cold isopropanol are added. The salt concentration of the solution is brought to 3 M by adding a calculated amount of a saturated sodium chloride solution and the cooled solution is kept at cold temperature (about 4°C) overnight. The precipitate formed is separated by centrifugation at 10,000 rpm for 20 minutes and the purity of the product is controlled by overnight dialysis and

subsequent ^{1}H-NMR analysis in order to ascertain that signals in the region below 1.5 ppm are absent. If necessary, the procedure of dissolution in water containing 4M NaCl and precipitation with isopropanol is repeated. The precitate is dissolved in water and ultrafiltrated on a Miniplate membrane Millipore with a 10,000 D cut off till disappearance of the salts. A K5 is obtained having a purity of at least 99% and giving a ^{1}H-NMR spectrum in which traces of lipophilic impurities in the region below 1.5 ppm are undetectable.

PREPARATION II

Preparation of a K5-N-sulfate

(i) N-deacetylation

One gram of pure K5 polysaccharide prepared as described in PREPARATION I are dissolved with 100 ml of 2 N sodium hydroxide and the solution thus prepared is kept at 60°C for 24 hours. The solution is brought to room temperature and then to neutral pH with 6N hydrochloric acid.

(ii) N-sulfation

To the solution containing the deacetylated K5, kept at 40°C, 1.6 g of sodium carbonate and, subsequently, 1.6 g of pyridine.sulfur trioxide are added in 4 hours. At the end of the reaction, after 24 hours, the solution is brought to room temperature and then to pH 6.5-7 with a 5% solution of hydrochloric acid. The K5-N-sulfate thus obtained is purified from salts by diafiltration using a spiral membrane of 1,000 D (Prepscale Cartridge-Millipore). The process is ended when the conductivity of the permeate is below 1,000 μS, preferably below 100 μS. The intradialysis is reduced till a polysaccharide concentration of 10% using the same in concentration dialysis system. The concentrated solution is freeze dried. The ^{13}C-NMR spectrum of the K5-N-sulfate does not show any signal of residual N-acetyl or NH_2 groups.

PREPARATION III

LMW- K5-N-sulfate

The product obtained in PREPARATION II is depolymerized by the degradation method with nitrous acid and subsequent reduction of the formed aldehyde. One gram of K5-N-sulfate is dissolved in 200 ml of distilled water and 480 mg of sodium nitrite dissolved in 240 ml of distilled water are added thereinto. The solution is then cooled to 4°C and the pH is brought to 2 with 0.1N HCl and maintained for 30 minutes. At the end of the reaction the solution is brought to pH 7 with 0.1N NaOH and then to room temperature. The solution is then added with 450 mg of $NaBH_4$ and left to react for 4 hours. The excess NaBH4 is eliminated with HCl by bringing the

pH to 5-6. The product, neutralized with 0.1 M NaOH, is recovered by precipitation with 3 volumes of acetone at 4°C, filtration with a glass funnel and dried at 40°C in vacuum oven. Thus, 900 mg of a LMW-K5-N-sulfate having a mean molecular weight of about 2,000 are obtained.

PREPARATION IV

K5-N,O-oversulfate

One gram of K5-N-sulfate obtained as described in PREPARATION II is dissolved in 100 ml of deionized water and the solution is cooled to 10°C with a cooling bath and then passed through a cationic exchange resin IR 120 H^+ or equivalent (50-200 ml). Both the column and the reservoir of the eluate are maintained at 10°C. After the passage of the solution containing the sample, the resin is washed with deionized water till the pH of the permeate is higher than 6 (about 3 volumes of deionized water). The acidic solution is brought to neutrality with tetrabutylammonium hydroxide (15% aqueous solution), then reduced to the minimum volume and freeze dried. The tetrabutylammonium salt is dissolved in 40 ml of DMF and added with 3.5 g of adduct pyridine.SO_3 in solid form. The solution is kept at 50°C for 24 hours.. At the end of the reaction the solution is cooled to room temperature and added with 3 volumes of a saturated sodium chloride solution in acetone till complete precipitation. The precipitate is separated from the solvent by filtration, solubilized with the minimum amount of deionized water (for example 100 ml) and added with sodium chloride to obtain a 0.2M solution. The solution is brought to pH 7.5-8 with 2N sodium hydroxide and added with 2 volumes of acetone till complete precipitation. The precipitate is separated from the solvent by filtration. The solid obtained is solubilized with 100 ml of deionized water and purified from residual salts by ultrafiltration using a spiral membrane of 1,000 D cut-off (prepscale cartridge-Millipore).

The solution containing the O-sulfated product is treated for the N-sulfation as previously described in step (ii) of PREPARATION II. The product has a sulfate to carboxyl ratio of 3.87 measured by conductimetry according to Casu et al. and a mean molecular weight of 20,000 measured by molecular exclusion HPLC.

Example 1

One gram of K5-N,O-oversulfate obtained as described in PREPARATION IV is dissolved in 200 ml of deionized water and thermostated to 4°C. Then 230 mg of sodium nitrite dissolved in an aqueous solution at the concentration of 0.2% are added. The solution containing the K5-N,O-oversulfate and sodium nitrite, kept at

4°C, is brought to pH 2 by addition of 0.1N HCl cooled to 4°C. The solution is left to react under slow stirring for 30 minutes, then neutralized with 0.1N NaOH. The solution containing the LMW-K5-N,O-oversulfate so obtained, consisting of a mixture of chains in which the preponderant species is a decasaccharide of formula I° in which m is 4 and X' is formyl, is brought to room temperature and treated with 250 mg of sodium borohydride dissolved in 50 ml of water and left to react for 4 hours. The excess sodium borohydride is eliminated by bringing the pH to about 5 with 0.1N HCl and left for further 2 hours. At the end the solution is neutralized with 0.1N NaOH and the product is recovered by precipitation with acetone after concentration of the product by evaporation under reduced pressure. The LMW-K5-N,O-oversulfate so obtained shows characteristics of sulfation similar to those of the K5-N,O-oversulfate starting material, a mean molecular weight of about 4,250 measured by viscosimetry and consists of a mixture of chains in which the preponderant species is a decasaccharide of formula I' in which m is 4 and X' is CH2OH.

Example 2

One gram of K5-N,O-oversulfate obtained as described in PREPARATION IV is treated as described in EXAMPLE 1 using 200 mg of sodium nitrite. The product obtained shows characteristics of sulfation similar to those of the K5-N,O-oversulfate starting material, a mean molecular weight of about 5,000 measured by viscosimetry and consists of a mixture of chains in which the preponderant species is a dodecasaccharide of formula I' in which m is 5 and X' is CH_2OH.

Example 3

One gram of K5-N,O-oversulfate obtained as described in PREPARATION IV is treated as described in Example 1 using 160 mg of sodium nitrite. The LMW-K5-N,O-oversulfate thus obtained shows characteristics of sulfation similar to those of the K5-N,O-oversulfate starting material, a mean molecular weight of about 6,000 measured by viscosimetry and consists of a mixture of chains in which the preponderant species is a tetradecasaccharide of formula I' in which m is 6 and X' is CH_2OH.

Example 4

(a) Tetrabutylammonium salt of LMW-K5-N-sulfate

A solution of 500 mg of LMW-K5-N-sulfate obtained as described in PREPARATION III in 50 ml of water is thermostated to 4°C, then passed through a IR 120 H^+ ionic exchange resin preconditioned with water at 4°C. The eluate

obtained, consisting of 125 ml of a solution at a pH of about 2, is neutralized with a 15% solution of tetrabutylammonium hydroxide and left at room temperature for one hour, by maintaining the pH at 7 by addition of a 15% tetrabutylammonium hydroxide solution and finally is freeze dried. One gram of the tetrabutylammonium salt of LMW-K5-N-sulfate is obtained.

(b) LMW-K5-amine-O-oversulfate

A solution containing one gram of the so obtained salt in 20 ml of dimethylformamide is kept at 55°C and treated with 20 ml of dimethylformamide containing 1.7 g of pyridine.SO_3 adduct. The reaction at 55°C is performed overnight, then 40 ml of water are added to the mixture. After neutralization with 1N NaOH, the product is precipitated with 3 volumes of saturated NaCl solution in acetone and kept at 4°C overnight. The precipitate is recovered by filtration on a G4 glass thunnel and then ultrafiltrated with a TFF Millipore system with 1,000 D cut off and dried at reduced pressure. Thus, there are obtained 683.2 mg of LMW-K5-amine-O-oversulfate consisting of a mixture of chains in which the preponderant species is a decasaccharide of formula III" wherein m is 4, for a degree of sulfation of about 2.9.

(c)LMW-K5-N,O-oversulfate

To a solution of 500 mg of the LMW-K5-amine-O-oversulfate obtained in step (b) in 30 ml of water, 800 mg of sodium carbonate are added, then 800 mg of pyridine.SO_3 adduct in solid form are added stepwise in 4 hours to the so obtained solution. The reaction mixture is kept at 55°C overnight, then the reaction is stopped by bringing its pH to 7 by addition of 0.1 N HCl. After ultrafiltration on a 1,000 D membrane, 3 volumes of a saturated solution of NaCl in acetone are added and the precipitate is recovered by centrifugation at 5,000 rpm for 5 minutes. Thus, there are obtained 502 mg of a LMW-K5-N,O-oversulfate having a mean molecular weight of about 4,100 measured by viscosimetry, consisting of a mixture of chains in which the preponderant species is a decasaccharide of formula I" wherein m is 4, with a degree of sulfation of about 3.9.

Example 5

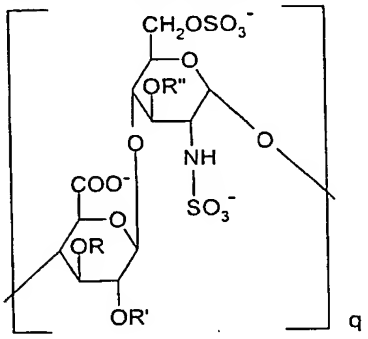
One gram of LMW-K5-N-sulfate obtained as described in PREPARATION III is submitted to steps (a) and (b) as described in Example 4. The LMW-K5-amine-O-oversulfate is recovered by precipitation with 3 volumes of a saturated solution of NaCl in acetone, dissolution of the precipitate obtained in water, ultrafiltration on a 1,000 D cut-off membrane and freeze drying. The so obtained product is a LMW-

K5-amine-O-oversulfate having a mean molecular weight of about 3,600 measured by viscosimetry and consists of a mixture of chains in which the preponderant species is a decasaccharide of formula III" wherein m is 4.

CLAIMS

1. A LMW-K5-N,O-oversulfate having a mean molecular weight of from about 3,000 to about 6,000 and a degree of sulfation of from 3.2 to 4.

2. The LMW-K5-N,O-oversulfate of claim 1 consisting of a mixture of chains in which the preponderant species has the formula I



(I)

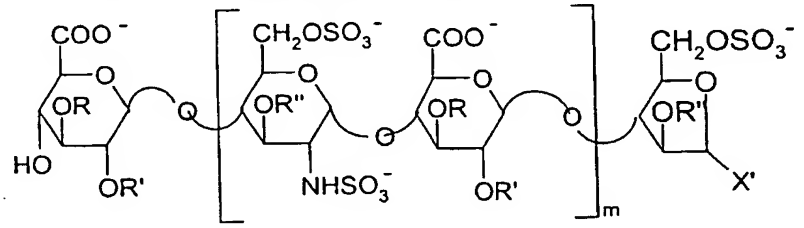
wherein q is 4, 5, 6, 7, or 8, R, R' and R" represent hydrogen or a SO3- group, for a degree of sulfation of from 3.2 to 4, and the corresponding cation is a chemically or pharmaceutically acceptable one.

3. The LMW-K5-N,O-oversulfate of claim 1 or 2 having a mean molecular weight of 3,750-4,250

4. The LMW-K5-N,O-oversulfate of claim 1 or 2 having a mean molecular weight of 4,750-5,250.

5. The LMW-K5-N,O-oversulfate of claim 1 or 2 having a mean molecular weight of 5,750-6,250.

6. The LMW-K5-N,O-oversulfate of claim 1 or 2 consisting of a mixture of chains in which the preponderant species is a compound of formula I°



(I°)

in which R, R' and R" represent hydrogen or SO_3^-, X' represents a formyl or hydroxymethyl group, for a degree of sulfation of from 3.2 to 4, m represents 4, 5 or 6 and the corresponding cation is a chemically or pharmaceutically acceptable one.

7. The LMW-K5-N,O-oversulfate according to claim 6, having a mean molecular weight of 3,750-4,250.

8. The LMW-K5-N,O-oversulfate according to claim 6, having a mean molecular weight of 4,750-5,250.

9. The LMW-K5-N,O-oversulfate according to claim 6, having a mean molecular weight of 5,750-6,250

10. A LMW-K5-N,O-oversulfate according to one of claims from 1 to 9, having a degree of sulfation of from 3.5 to 4.

11. A LMW-K5-N,O-oversulfate according to one of claims from 1 to 9, having a degree of sulfation of from 3.5 to 3.9.

12. A process for the preparation of a LMW-K5-N,O-oversulfate having a degree of sulfation of from 3.2 to 4, which comprises

(a) treating a LMW-K5-N-sulfate obtained by nitrous depolymerization of a K5-N-sulfate and subsequent reduction, in its acidic form, with a tertiary amine or quaternary ammonium hydroxide, letting the reaction mixture to stand for a period of time of 30-60 minutes by maintaining the pH of the solution at 7 and isolating its salt with said organic base;

(b) treating said tertiary amine or quaternary ammonium salt of said polysaccharide with an O-sulfating agent under O-oversulfation conditions;

(c) treating the product thus obtained with a N-sulfating agent and isolating the LMW-K5-N,O-oversulfate thus obtained.

13. A process according to claim 12, which comprises

(i) submitting a K5-N-sulfate to a nitrous depolymerisation followed by a reduction, for example by sodium borohydride;

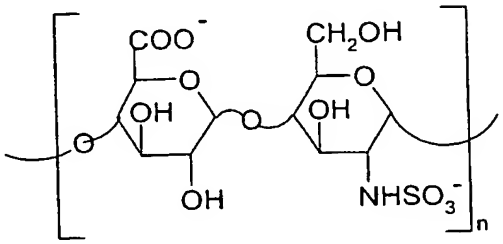
(ii) treating a LMW-K5-N-sulfate, in its acidic form, with a tertiary amine or quaternary ammonium hydroxide, letting the reaction mixture to stand for a period of time of 30-60 minutes, whereby the pH of the solution is maintained at 7, and isolating the corresponding tertiary amine or quaternary ammonium salt;

(iii) treating said tertiary amine or quaternary ammonium salt of said LMW-K5-N-sulfate with an O-sulfation reactant under O-oversulfation conditions;

(iv) treating the product thus obtained with a N-sulfating agent and isolating the obtained LMW-K5-N,O-oversulfate.

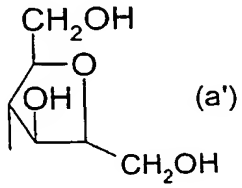
14. A process according to claim 12, wherein said reduction is carried out with sodium borohydride.

15. A process according to claim 12, wherein a LMW-K5-N-sulfate consisting of a mixture of chains in which at least 90% of said chains has the formula II



(II)

wherein n is an integer from 2 to 20, containing a 2,5 anhydrommanitol unit of structure (a')



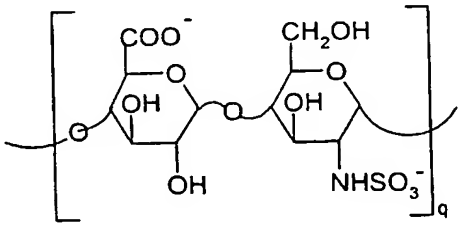
(a')

at the reducing end of the majority of chains in said mixture of chains, and the corresponding cation is a chemically and pharmaceutically acceptable one, is used as starting material.

16. A process according to one of claims 12 to 14, wherein said K5-N-sulfate starting material is free of lipophilic substances.

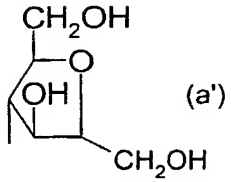
17. A process according to one of claims from 12 and 14 to 16, wherein the LMW-K5-N-sulfate starting material is used in the form of its sodium salt.

18. A process according to one of claims 12 and 14 to 16, wherein said K5-N-sulfate starting material consists of a mixture of chains in which the preponderant species is a compound of formula II'



(II')

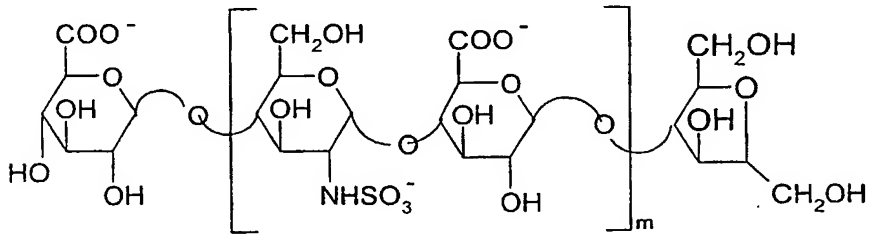
in which q is 4, 5, 6, 7, or 8, containing a 2,5 anhydrommanitol unit of structure (a')



(a')

at the reducing end of the majority of the chains in said mixture of chains, and the corresponding cation is a chemical or pharmaceutically acceptable one.

19. A process according to one of the claims 12 and 14 to 18, wherein said K5-N-sulfate starting material consists of a mixture of chains in which the preponderant species is a compound of formula II"



(II")

in which m represents 4, 5 or 6 and the corresponding cation is a chemically or pharmaceutically acceptable one.

20. A process according to one of the claims 12 to 19, wherein the LMW-K5-N,O-oversulfate is obtained in its sodium salt form and optionally transformed into another chemically or pharmaceutically acceptable salt.

21. A LMW-K5-amine-O-oversulfate obtainable by the steps (a) and (b) of the process according one of claims from 12 to 20, or a chemically or pharmaceutically acceptable salt thereof.

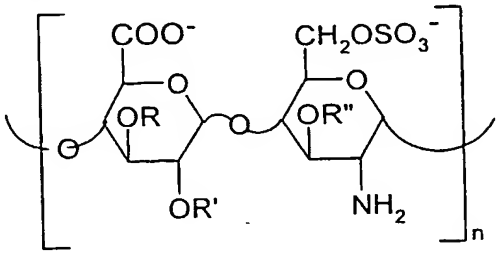
22. A LMW-K5-amine-O-oversulfate having a degree of sulfation of from 2.2 to 3 or one of its chemically or pharmaceutically acceptable salts.

23. A LMW-K5-amine-O-oversulfate according to one of claims 21 and 22, having a mean molecular weight of from about 3,500 to about 11,000 or a chemically or pharmaceutically acceptable salt thereof.

24. A LMW-K5-amine-O-oversulfate according to one of claims from 21 to 23 having a degree of sulfation of from 2.2 to 3 and a molecular weight of from 3,500 to 5,200.

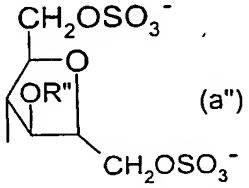
25. A LMW-K5-amine-O-oversulfate according to one of claims from 21 to 24, substantially free of N-acetyl groups.

26. A LMW-K5-amine-O-oversulfate according to one of claims from 20 to 24 consisting of a mixture of chains in which at least 90% of said chains has the formula III



(III)

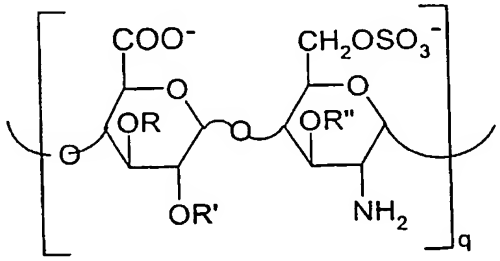
in which R, R' and R'' represent hydrogen or a SO3- group, n is a number between 2 and 20, containing a sulfated 2,5-anhydrommanitol unit of structure (a'')



(a")

at the reducing end of the majority of the chains in said mixture of chains, for a degree of sulfation of from 2.2 to 3 and the corresponding cation is a chemically or pharmaceutically acceptable one.

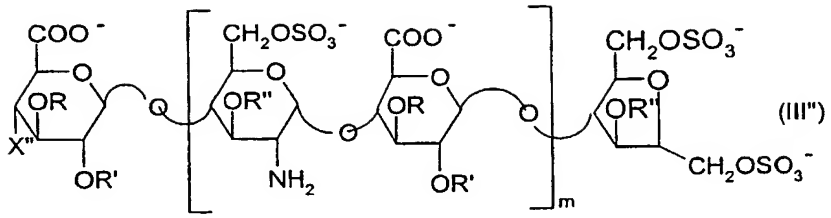
27. The LMW-K5-amine-O-oversulfate of claim 26, consisting of a mixture in which the preponderant species is a compound of formula III'



(III')

in which q is 4, 5, 6, 7, or 8 and the corresponding cation is a chemically or pharmaceutically acceptable one.

28. The LMW-O-oversulfated-K5 amine of claim 27, consisting of a mixture in which the preponderant species is a compound of formula III''

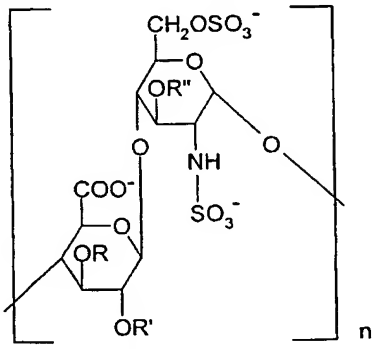


(III")

in which R, R' and R" are hydrogen or SO_3^-, X" is OH or OSO_3^-, for a degree of sulfation of from 2.2 to 3, m is 4, 5 or 6 and the corresponding cation is a chemically or pharmaceutically acceptable one.

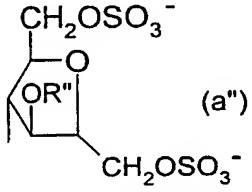
29. A LMW-K5-N,O-oversulfate obtainable according to the process of the claims from 12 to 20.

30. A LMW-K5-N,O-oversulfate consisting of a mixture of chains in which at least 90% of said chains has the structure I'



(I')

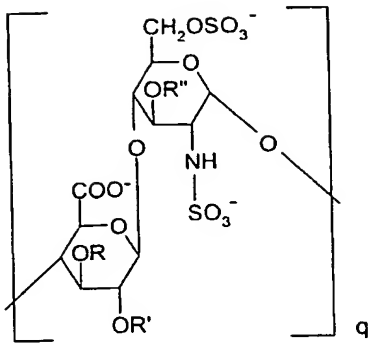
in which n is an integer from 2 to 20, R, R' and R" represent hydrogen or a SO_3^- group, and in which the reducing end of the majority of said chains has the structure (a'')



(a")

in which R" is hydrogen or a SO_3^- group, for a degree of sulfation of from 3.2 to 4 and the corresponding cation is a chemically or pharmaceutically acceptable one.

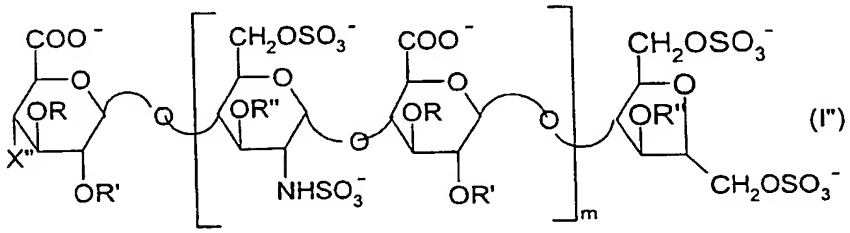
31. The LMW-K5-N,O-oversulfate of the claim 30, consisting of a mixture of chains in which the preponderant species is a compound of formula I



(I)

wherein q is 4, 5, 6, 7, or 8 and the corresponding cation is a chemically or pharmaceutically acceptable one.

32. The LMW-K5-N,O-oversulfate of claim 31, consisting of a mixture in which the preponderant species is a compound of formula I"



(I")

in which m is 4, 5, 6, R, R' and R" are hydrogen or SO_3^-, for a degree of sulfation of from 3.2 to 4 and the corresponding cation is a chemically or pharmaceutically acceptable one.

33. A LMW-K5-N,O-oversulfate according to one of the claims from 29 to 32 wherein said cation is the ion of an alkaline metal, an alkaline-earth metal, ammonium, tetra(C_1-C_4)alkylammonium, aluminum and zinc.

34. The LMW-K5-N,O-oversulfate of claim 33, wherein said cation is the sodium, calcium or tetrabutylammmonium ion.

35. A LMW-K5-N,O-oversulfate according to one of claims from 29 to 34, having a degree of sulfation of from 3.5 to 4.

36. A LMW-K5-N,O-oversulfate according to one of the claims from 29 to 34, having a degree of sulfation of from 3.5 to 3.9.

37. A process for the preparation of LMW-K5-N-sulfates and of their chemically or pharmaceutically acceptable salts, which comprises submitting a K5-N-sulfate to a controlled nitrous depolymerization optionally followed by a redution and isolating the product thus obtained.

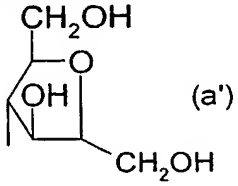
38. A process according to claim 37, wherein said K5-N-sulfates are isolated as sodium salts which is optionally converted into another chemically or pharmaceutically acceptable salt.

39. A process according to claim 38, wherein said other salt is that of an alkaline metal, an alkaline-earth metal, ammonium, tetra(C_1-C_4)lkylammonium, aluminum and zinc.

40. A process according to claim 39, wherein said other salt is that of sodium, calcium or tetrabutylammonium.

41. A LMW-K5-N-sulfate obtainable according to anyone of claims 37 to 40.

42. A LMW-K5-N-sulfate obtained according to the process of claims 37 to 40 containing a 2,5 anhydrommanitol unit of structure (a')



(a')

at the reducing end of the majority of chains in said mixture of chains.

43. A LMW-K5-N-sulfate or a chemically or pharmaceutically acceptable salt thereof.

44. A LMW-K5-N-sulfate according to claim 43, consisting of a mixture of chains in which at least 90% of said chains has a mean molecular weight of from about 1,500 to about 7,500.

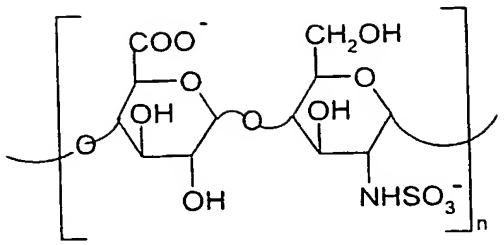
45. A LMW-K5-N-sulfate according to claim 44, having a molecular weight distribution from about 1,000 to about 10,000.

46. A LMW-K5-N-sulfate according to claims from 41 to 45, containing from 0 to no more than 5% acetyl groups.

47. A LMW-K5-N-sulfate according to claims from 41 to 46, having a mean molecular weight of from about 2,000 to about 4,000.

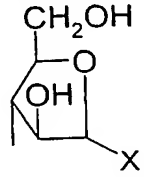
48. A LMW-K5-N-sulfate according to claims from 41 to 46, having a mean molecular weight of from about 4,000 to about 7,500.

49. A LMW-K5-N-sulfate according to claims from 41 to 46, consisting of a mixture of chains in which at least 90% of said chains has the formula II



(II)

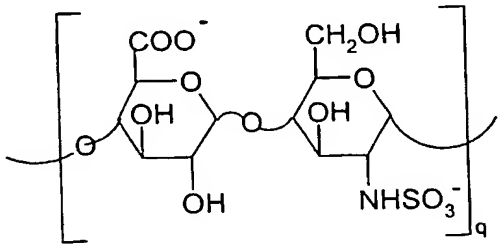
wherein n is a number from 2 to 20, containing an unit of structure



(a)

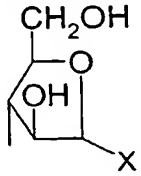
in which X represents formyl or hydroxymethyl, in the majority of said chain and the corresponding cation is a chemically or pharmaceutically acceptable one.

50. A LMW-K5-N-sulfate according to claims from 41 to 46, consisting of a mixture of chains in which the preponderant species is a compound of formula II'



(II')

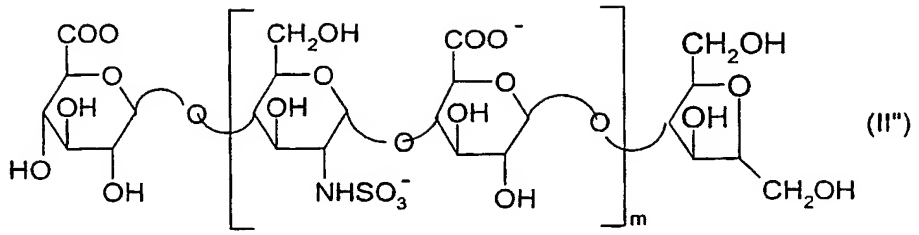
wherein q is 4, 5, 6, 7 or 8, containing an unit of structure



(a)

in which X represents formyl or hydroxymethyl, in the majority of said chain and the corresponding cation is a chemically or pharmaceutically acceptable one.

51. A LMW-K5-N-sulfate according to claims from 41 to 46, consisting of a mixture of chains in which the preponderant species is a compound of formula II"



(II")

wherein X represents formyl or hydroxymethyl, m represents 4, 5, or 6 and the corresponding cation is a chemically or pharmaceutically acceptable one.

52. A LMW-K5-N-sulfate according to claims from 49 to 51 wherein, in the structure (a), X is hydroxymethyl.

53. A LMW-K5-N-sulfate according to claims from 41 to 52, wherein said salt or cation is that of an alkaline metal, alkaline-earth metal, ammonium, tetra(C_1-C_4)alkylammonium, aluminum and zinc.

54. A LMW-K5-N-sulfate according to claims from 41 to 52, wherein said salt or cation is that of sodium, calcium or tetrabutylammonium.

55. A pharmaceutical composition comprising, as an active ingredient, a LMW-K5-N,O-oversulfate according to one of claims from 1 to 11 or from 28 to 35, in admixture with a pharmaceutical carrier.

INTERNATIONAL SEARCH REPORT

Internat[redacted]pplication No
PCT/I[redacted]/02347

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C08B37/00 C08B37/08 C08B37/10 A61K31/726 A61K31/727
A61K31/737

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols) .
IPC 7 C08B

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
WPI Data, PAJ, CHEM ABS Data, EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 02 068477 A (ORESTE PASQUA ANNA ;ZOPPETTI GIORGIO (IT)) 6 September 2002 (2002-09-06) page 8, line 17 -page 11, line 2 ---	1-55
P,X	WO 03 011307 A (VICENZI ELISA ;POLI GUIDO (IT); ORESTE PASQUA ANNA (IT); SAN RAFFA) 13 February 2003 (2003-02-13) page 7, line 2 -page 11, line 14 page 17, line 4 -page 19, line 6 ---	1-55
P,X	WO 02 083155 A (PRESTA MARCO ;ORESTE PASQUA ANNA (IT); UNI DEGLI STUDI DI BRESCIA) 24 October 2002 (2002-10-24) page 11, line 28 -page 12, line 27 ---	1-55

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

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"O" document referring to an oral disclosure, use, exhibition or other means

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 October 2003	05/11/2003

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Mazet, J-F .

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	EP 0 544 592 A (SANOFI ELF) 2 June 1993 (1993-06-02) cited in the application abstract; claims page 21, line 42 -page 22, line 60 examples	1-55
A	WO 01 72848 A (CIPOLLETTI GIOVANNI ;PASQUA ORESTE (IT); INALCO SPA (IT); ZOPPETTI) 4 October 2001 (2001-10-04) cited in the application page 9, line 30 -page 10, line 4 claims; examples	1-55
A	US 2002/062019 A1 (ORESTE PASQUA ET AL) 23 May 2002 (2002-05-23) cited in the application the whole document	1-55
A	LEALI D ET AL: "FIBROBLAST GROWTH FACTOR-2 ANTAGONIST ACTIVITY AND ANGIOSTATIC CAPACITY OF SULFATED ESCHERICHIA COLI K5 POLYSACCHARIDE DERIVATIVES" JOURNAL OF BIOLOGICAL CHEMISTRY, AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, BALTIMORE, MD, US, vol. 276, no. 41, 12 October 2001 (2001-10-12), pages 37900-37908, XP001077702 ISSN: 0021-9258 cited in the application	
A	WO 97 43317 A (CIPOLLETTI GIOVANNI ;PASQUA ORESTE (IT); INALCO SPA (IT); ZOPPETTI) 20 November 1997 (1997-11-20) cited in the application page 6, line 27 -page 7, line 2 claims	1
A	NAGGI A ET AL: "TOWARD A BIOTECHNOLOGICAL HEPARIN THROUGH COMBINED CHEMICAL AND ENZYMATIC MODIFICATION OF THE ESCHERICHIA COLI K5 POLYSACCHARIDE" SEMINARS IN THROMBOSIS AND HEMOSTASIS, STUTTGART, DE, vol. 27, no. 5, 2001, pages 437-443, XP008004483 ISSN: 0094-6176 the whole document	1

INTERNATIONAL SEARCH REPORT

Interna pplication No
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